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Islet-Derived CD4 T Cells Targeting Proinsulin in Human Autoimmune Diabetes

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Type 1 diabetes results from chronic autoimmune destruction of insulin-producing β-cells within pancreatic islets. Although insulin is a critical self-antigen in animal models of autoimmune diabetes, due to extremely limited access to pancreas samples, little is known about human antigenic targets for islet-infiltrating T cells. Here we show that proinsulin peptides are targeted by islet-infiltrating T cells from patients with type 1 diabetes. We identified hundreds of T cells from inflamed pancreatic islets of three young organ donors with type 1 diabetes with a short disease duration with high-risk HLA genes using a direct T-cell receptor (TCR) sequencing approach without long-term cell culture. Among 85 selected CD4 TCRs tested for reactivity to preproinsulin peptides presented by diabetes-susceptible HLA-DQ and HLA-DR molecules, one T cell recognized C-peptide amino acids 19–35, and two clones from separate donors responded to insulin B-chain amino acids 9–23 (B:9–23), which are known to be a critical self-antigen–driving disease progress in animal models of autoimmune diabetes. These B:9–23–specific T cells from islets responded to whole proinsulin and islets, whereas previously identified B:9–23 responsive clones from peripheral blood did not, highlighting the importance of proinsulin-specific T cells in the islet microenvironment.

Type 1 diabetes results from chronic T cell–mediated destruction of insulin-producing β-cells within pancreatic islets (1). Type 1 diabetes is increasing in incidence and is often predictable by screening for autoantibodies directed to islet antigens in peripheral blood (2,3). Although several clinical trials using preparations of insulin (subcutaneous, oral, and intranasal) to delay or prevent diabetes onset have been completed, the disease is not yet preventable (4–7). Better understanding the T-cell immune response to insulin in the target organ is required to improve outcomes.

Much of our understanding regarding disease pathogenesis comes from studying animal models of autoimmune diabetes. In particular, the murine model of spontaneous autoimmune diabetes, the nonobese diabetic (NOD) mouse, has significant similarities to human disease with homologous MHC class II genes conferring risk (8,9), the development of insulin autoantibodies prior to diabetes onset, and T-cell infiltration within pancreatic islets (10). Having the ability to study immune cells within the target organ of the NOD mouse led to the discovery that insulin is a critical autoantigen determining diabetes development (11–14). Notably, many murine islet–derived T cells recognize a fragment of insulin, B-chain amino acids 9–23

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(B:9–23) (11). By mutating a single amino acid within the B chain of insulin (B16 tyrosine to alanine), insulin loses immunogenicity and mice remain euglycemic without T-cell infiltration in islets (14), which is not the case for other islet antigens (e.g., GAD, islet antigen-2, and islet-specific glucose-6-phosphatase catalytic subunit–related protein) (15–17).

Given the importance of insulin as a self-antigen in the NOD mouse, T-cell responses to proinsulin epitopes have been explored in human disease with several groups isolating T-cell clones from the peripheral blood (18–22). However, compared with animal models, little is known about antigens targeted by islet-infiltrating T cells in human disease because of the anatomic location and difficulty in obtaining these tissues from patients with type 1 diabetes. This has resulted in very few studies examining T-cell reactivity within pancreatic lymph nodes and islets in human patients. Kent et al. (23) cloned CD4 T cells from pancreatic lymph nodes of three patients with established type 1 diabetes 10 years ago, identifying clones from two patients responding to insulin A-chain amino acids 1–15. Recently, Mannering and colleagues (24) established and analyzed T-cell clones derived from islets of a single organ donor with type 1 diabetes, which identified six epitopes within the C-peptide portion of proinsulin as CD4 T-cell targets.

It is essential to understand the interplay between T cells in the pancreas and the major genetic determinants of disease development (i.e., HLA genes) to provide a framework to improve prevention efforts for type 1 diabetes. To acquire direct insights into target organ-specific T cells, we analyzed CD4 and CD8 T cells from inflamed pancreatic islets of three young organ donors having type 1 diabetes with the high-risk HLA genes. T cells were directly isolated without long-term culture to recapitulate T-cell receptor (TCR) repertoires in pancreatic islets, followed by single-cell sorting and TCR sequencing of individual cells. We provide evidence of islet-infiltrating T cells targeting proinsulin, including insulin B:9–23, in the pathogenesis of human type 1 diabetes.

RESEARCH DESIGN AND METHODS

Study Approval

The donation of tissue samples from organ donors was approved by the institutional review boards for each university involved in the studies. Maintenance and use of all the mouse strains were approved by the Institutional Animal Care and Use Committee at the University of Colorado.

Organ Donors With Type 1 Diabetes

Organ donors with type 1 diabetes were identified through the Network for Pancreatic Organ Donors with Diabetes (nPOD) (http://www.jdrfnpod.org/) (25) or the Integrated Islet Distribution Program (IIDP) (https://iidp.coh.org/Default.aspx). Type 1 diabetes–associated autoantibodies (insulin autoantibody, GAD antibody, islet antigen-2 antibody, zinc transporter 8 antibody) were measured in serum isolated upon death by radioimmunoassay as previously described (26), and genotyping for HLA was performed using linear arrays of immobilized sequence-specific oligonucleotides (27). Insulin variable number tandem repeat (VNTR) genotypes were determined as previously described (28).

Histological Analysis

Tissues from pancreas tail and head were isolated, fixed by formalin, and embedded in paraffin blocks through the nPOD Organ Processing and Pathology Core (29) (donors 6323 and 6342). Small portions of pancreas tissue from donor 69 were processed to formalin-fixed paraffin blocks by IIDP. Immunohistochemistry was performed on serial sections from each patient and were double stained for Ki-67 and insulin, CD3, and glucagon (30).

Isolation and Single-Cell Sorting of T Cells From Pancreatic Islets

Pancreatic islets were isolated by standard procedures (https://iidp.coh.org/sops.aspx) at either the University of Pennsylvania or the University of Pittsburgh. Approximately 500 hand-picked islets or islet equivalents were put in 24-well culture plates containing RPMI medium supplemented with penicillin-streptomycin, human AB^+ serum, interleukin (IL)-2 (30 units/mL), and IL-15 (10 ng/mL) followed by culture at 37°C with 5% CO_2 for 1–4 days (Table 1). For dispersion to single cells, islets were treated with collagenase type 1 and DNase I, or with Liberase DL (donor 6342), or were mechanically separated by pipetting (donor 69). The dispersed single islet cells were then stained with phycoerythrin-conjugated anti-CD3e (UCHT1), allophycocyanin-conjugated anti-CD4 (RPA-T4), fluorescein isothiocyanate–conjugated anti-CD8 (RPA-T8), and allophycocyanin-cyanine 7–conjugated anti-CD19 (HIB19) antibodies along with 4′6-diamidino-2-phenylindole, followed by flow cytometric cell sorting on MoFlo Astrios EQ sorter using an index-sorting approach. The sorting strategy is shown in Supplementary Fig. 1. Single CD4 and CD8 T cells were sorted in each well of 96-well plates.

Single-Cell TCR Sequencing

Single cells were lysed by the Nonidet P-40 detergent solution and heat treated at 65°C for 10 min, and were subject to the reverse-transcriptase reaction using SuperScript III Reverse Transcriptase in the presence of random hexamers and constant region–specific primers (TRAC-R and TRBC-R2). The multiplex PCR used to amplify TCR α- and β-chain genes was performed separately using a mixture of primers targeting individual variable region genes and the constant region–specific primer (TRAC-R3 or TRBC-R2). The variable region–specific primers are conjugated with a universal oligonucleotide, and thus a primer specific for the universal sequence (Illumina-Short) along with the nested constant region–specific primer (TRAC-R5 or TRBC-R7) were used for the following PCR to further amplify TCR genes. The PCR products were then subject to the final PCR using

Table 1—Characteristics of organ donors with type 1 diabetes

	nPOD donor 6323	nPOD donor 6342	nPOD donor 69
Sex	Female	Female	Female
Age (years)	19	14	6
Diabetes duration (years)	6	2	3
Cause of death	Anoxia	Anoxia	Anoxia
Islet autoantibodies			
mIAA (Index) (cutoff: 0.010)	0.001	0.220	0.061
GADA (NIDDK units) (cutoff: 20)	169	5	5
IA-2A (NIDDK units) (cutoff: 5)	19	76	0
ZnT8A (Index) (cutoff: 0.030)	0.004	0.005	0.000
HLA allele			
A	01/25	02/68	02/26
B	08/18	40	35/50
C	Not tested	03	04/06
DRB1	0301/0401 (DR3/4)	0101/0401 (DR1/4)	0401/0701 (DR4/7)
DQA1	0501/0301	0101/0301	0301/0201
DQB1	0201/0302	0501/0302	0302/0202
Insulin VNTR	Class III/I	Class III/I	Class III/I

GADA, GAD antibody; IA-2A, islet antigen 2 antibody; mIAA, insulin autoantibody; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; ZnT8A, zinc transporter 8 antibody.

the additional nested constant region–specific primer (Illumina-TRAC-no or Illumina-TRBC-no) and the universal sequence-specific primer (Illumina-no) to conjugate with the adaptor oligonucleotides for high-throughput sequencing on an Illumina MiSeq sequencer. Each Illumina-TRAC, Illumina-TRBC, and Illumina-no primer contains a different identifier oligonucleotide to distinguish individual PCR products from both the 5′ and 3′ ends (Supplementary Tables 2 and 3). All primer sequences are listed in Supplementary Table 1.

Generation of Retroviral Vectors Encoding TCR Genes

Sequences determined by single-cell TCR Illumina sequencing lacked the beginning portion of variable region; therefore, the whole variable region gene sequences were determined from the RNA of each donor's spleen cells by 5′-rapid amplification of cDNA ends by PCR followed by sequencing on a 454 GS Junior System sequencer. α-Chain and β-chain genes connected by the porcine teschovirus-1 2A peptide gene were engineered into a murine stem cell virus–based retroviral vector carrying any of the following selection genes: neomycin-resistant, puromycin-resistant, green fluorescent protein, or yellow fluorescent protein genes (Supplementary Fig. 2 and Supplementary Table 4).

Generation of T-Cell Transductants

T-cell transductants were generated by retroviral transduction of the 5KC murine T-cell hybridoma cells lacking endogenous TCR expression (31). The 5KC cell line has been manipulated to express the human CD4 molecule having substitutions at positions 40 and 45 (Gln40Tyr and Thr45Trp) (32) to support interaction with HLA. Responsiveness of the manipulated 5KC cells was most robust compared with other host T-cell lines tested including those derived from humans, and therefore this 5KC cell line was chosen as host cells for TCR expression. To confirm the response without the mutations in CD4, 5KC cells expressing native human CD4 were also used for experiments shown in Supplementary Fig. 8. 5KC cells were spin-infected with replication-incompetent retroviruses that were produced from Phoenix cells transfected with the retroviral vectors and the pCL-Eco packaging vector. Cells transduced with retroviruses were then selected either by treatment with G418 sulfate or puromycin or flow cytometric cell sorting of fluorescence-positive cells according to the selection genes carried by the retroviral vectors.

Antigen-Presenting Cells Expressing HLA

The M12C3 murine B cell line (33) was used as antigen-presenting cells for initial peptide screening of T-cell transductants. The K562 human myelogenous cell line was also used for some of T-cell stimulation analysis as designated in the text or figure legends. M12C3 and K562 cells were spin-infected with replication-incompetent retroviruses produced from MSCV-based retroviral vectors containing the HLA-DR/DQ α- and β-chain gene fragments connected by the porcine teschovirus-1 2A peptide gene (Supplementary Fig. 2 and Supplementary Table 4). HLA α- and β-chain gene combinations are shown in Supplementary Table 5. Splenic B cells isolated from a DR3-DQ2/DR4-DQ8 heterozygous individual and nPOD donor 6342 were infected with Epstein Barr virus and were used as antigen-presenting cells to analyze nonspecific T-cell responders.

Screening of T-Cell Transductants for Responsiveness to Islet Antigens

T-cell transductants were cultured with or without overlapping preproinsulin peptides or islet antigen mimotopes in the presence of M12C3 cells expressing appropriate

HLA. For T-cell transductants derived from donor 6323, who had the DR3-DQ2/DR4-DQ8 genotype, each well contained mixtures of M12C3 cells expressing DR3 and those expressing DR4, those with DQ2 and DQ8, or those with DQ2-trans and DQ8-trans as antigen-presenting cells. For T-cell transductants from the other two donors, M12C3 cells expressing DR4 or DQ8 were added in each well as antigen-presenting cells. Coculture with anti-CD3 antibody (145–2C11) was assessed for each T-cell transductant as a positive control. IL-2 secretion in supernatants was measured by ELISA.

Characterization of Proinsulin-Reactive T-Cell Transductants

Three proinsulin-reactive T-cell transductants (GSE.6H9, GSE.20D11, and GSE.8E3) were further analyzed for dose responsiveness to cognate peptides and response to islet cells in the presence of antigen-presenting cell designated in the RESULTS section. T-cell transductants expressing other TCRs (i.e., T1D-3, T1D-10, Clone 5, and 489) generated using the same conditions were analyzed for comparison. IL-2 secretion in supernatants was measured by the highly sensitive ELISA (Meso Scale Diagnostics, LLC). All experiments were repeated at least three times.

Preparation of Human Dendritic Cells and Murine Spleen Cells

Monocytes from the peripheral blood of two separate patients with type 1 diabetes having the HLA DR3-DQ2/DR4-DQ8 genotype (DRB1*03:01-DQA1*05:01, DQB1*02:01/DRB1*04:01-DQA1*03:01, DQB1*03:02) were matured into human dendritic cells using granulocyte-macrophage colony-stimulating factor and IL-4. Spleens were harvested from NOD mice transgenic for DQ8 (Jax 006022 mice). Spleens were dispersed to single cells followed by red blood cell lysis.

Preparation of Murine and Human Islet Cell Antigens

Human islets were obtained from IIDP and treated with Liberase DL to disperse to single cells. Murine islets were harvested from immunocompromised NOD mice (Jax 001303, 004444, 023082 mice) followed by treatment with 0.25% trypsin-EDTA to disperse to single cells. Single islet cells (5×10^4 cells/well) were cocultured with DQ8-transgenic murine spleen cells for 6–16 h prior to adding T-cell transductants.

Statistics

Statistical tests were performed using GraphPad Prism 6.0 software. Levels of IL-2 responses were analyzed using an unpaired *t* test. A two-tailed *P* value of <0.05 is considered to be significant.

RESULTS

Organ Donor Description

We analyzed pancreas tissue and isolated pancreatic islet samples from three pediatric patients with type 1 diabetes, which are available from the nPOD Pilot Islet Study Group (25). All three had a relatively short duration of diabetes, islet autoantibodies, and high-risk HLA-DR-DQ genes, in particular the DR4-DQ8 haplotype (Table 1) (8). In addition, all donors were heterozygous for the second strongest genetic risk factor *INS-VNTR*, which correlates with differential proinsulin expression in the thymus versus pancreas (34).

Pancreas Histology

Histological analysis and islet isolation were performed for each patient. All three patients had residual islets identified by glucagon staining for α-cells. It is well appreciated that typical pancreata of patients with long-standing type 1 diabetes rarely express insulin within islets (30), whereas a number of islets in the studied donors 6323 and 6342 stained positive for insulin (Fig. 1*A* and *D*). T-cell islet infiltration was analyzed by staining for CD3, and all three patients showed evidence of insulitis (Fig. 1*B*, *E*, and *H*). These findings are consistent with a recent report (30) of patients with type 1 diabetes having islet lymphocytic infiltration with higher numbers of insulin-containing cells. The pancreas histology of donor 69 did not show insulin-containing cells but had CD3-positive cells around islets and in acinar tissue (Fig. 1*G* and *H*).

T Cells Infiltrating Pancreatic Islets

Pancreatic islets were isolated using standard islet isolation procedures. Islets were cultured in media containing cytokines to promote T-cell viability for 1–4 days (Table 2), followed by single-cell dispersion for flow-cytometric analysis (Supplementary Fig. 1). Short culture times were used to minimize the potential bias in the T-cell repertoire by selective outgrowth and to recapitulate the endogenous repertoires in the pancreas. The frequencies and ratios of CD4/CD8 T cells in the islets varied by donor (Table 1 and Fig. 1*C*, *F*, and *I*). Consistent with histological findings, large numbers of CD4 and CD8 T cells were detected in the samples from donors 6323 and 6342, which represented ~0.1–0.6% of the total islet cell suspensions analyzed, whereas very few islet-infiltrating T cells were found in the samples from donor 69. This low number of lymphocyte recovery from donor 69 may relate to a longer culture period prior to analysis but matched the peri-insulitis (rather than islet infiltration) seen in this donor.

TCR Sequences Expressed by Islet-Infiltrating T Cells

TCRs expressed by T cells recognize peptides presented by HLA molecules, thus determining the antigen specificity of T cells. To begin to define T-cell targets, CD4 and CD8 T cells were single-cell sorted from islets for TCR sequencing (Fig. 2*A*). We identified hundreds of TCR sequences (Table 2) with the majority of sequences being unique (Fig. 2*B*), indicating the diversity of polyclonal T cells within islets even years after disease onset. Yet, several TCRs were detected multiple times in the islets of each patient when a sufficient number of sequences was identified, in particular for CD8 T cells (Fig. 2*B*). Detecting

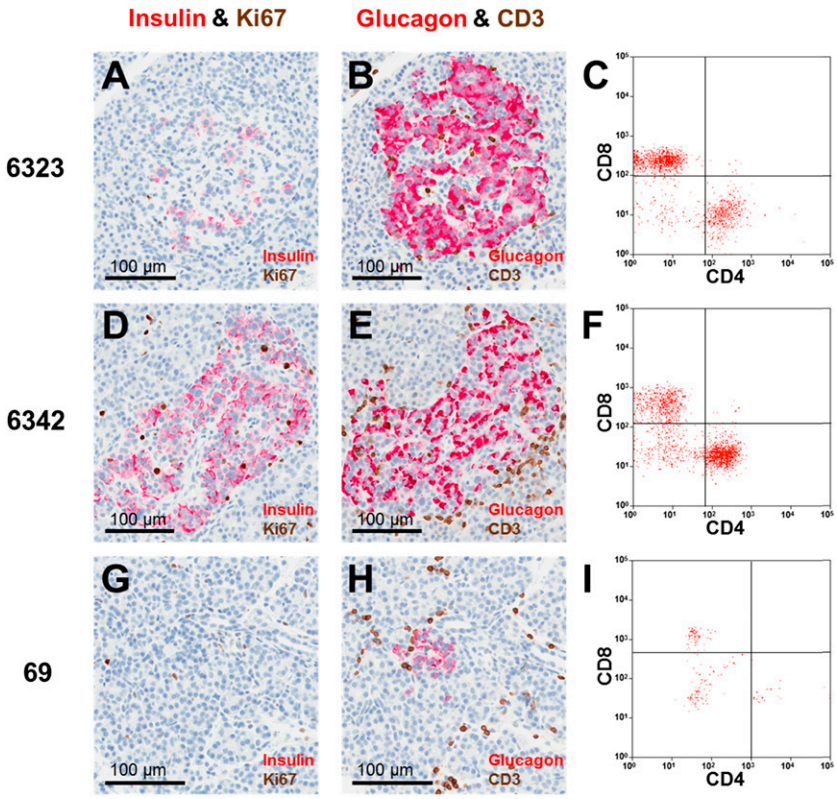


Figure 1—Pancreas histology and T-cell infiltration in pancreatic islets of the three studied organ donors with type 1 diabetes. Immunohistochemical staining of serial paraffin sections from the pancreata of the three organ donors. Donors 6323 (*A* and *B*), 6342 (*D* and *E*), and 69 (*G* and *H*) were stained for Ki67 (brown) and insulin (red) (*A*, *D*, and *G*) or CD3 (brown) and glucagon (red) (*B*, *E*, and *H*). All donors have islet-infiltrating CD3-positive cells, whereas donors 6323 and 6342 have residual insulin staining in islets. Flow-cytometric analysis of CD4 and CD8 T cells infiltrating the pancreatic islets of donors 6323 (*C*), 6342 (*F*), and 69 (*I*).

identical TCR sequences (including nucleotide sequences) repeatedly, despite the relatively short-term in vitro culture (donors 6323 and 6342), suggests oligoclonal T-cell expansion in vivo. It is noteworthy that the majority of repeatedly detected TCRs were found from two separate islet preparations in each donor (Fig. 2*C*). This indicates that clonally expanded T cells migrate to different islets within the pancreas.

T cells recognizing the same antigen can share a component of their TCRs. For example, identical α-chains paired with various β-chains recognize the same antigens, and vice versa. TCRs from the three patients commonly shared a component of their receptors, such as an identical α- or β-chain (Supplementary Table 6) or specific variable gene segments (Supplementary Fig. 3). In sum, there was a significant portion of T cells that was repeatedly detected or shared a component of the TCR, which conceivably was expanded upon specific antigen stimulation in the islet.

Response of Islet-Infiltrating T Cells to Islet Antigens

After analyzing TCR sequences, we aimed to determine peptide targets of the islet-infiltrating T cells. Using retroviral vectors encoding TCR genes, single TCRs were expressed on immortalized T cells devoid of endogenous TCR expression (31), thus creating TCR transductants. The use of immortalized T-cell transductants allowed us to screen identified receptors for antigen specificity and HLA restriction. For this report, we focused on CD4 T cells as the majority of the genetic risk within the HLA complex is restricted to amino acid polymorphisms in HLA-DQ-DR (8), which presents antigens to CD4 T cells. Criteria for selecting TCRs for the analysis of target antigens included the following: all seven CD4-derived TCRs for donor 69, those detected multiple times within a donor (donors 6323 and 6342), and those with frequently shared components of TCRs (Supplementary Tables 6 and 7). TCRs were expressed on murine T-cell hybridoma cells expressing mutated human CD4, which has two amino acid differences compared with native CD4, thereby providing more stable interaction with HLA. From the three patients, 90 TCR transductants were created with 85 successfully expressing receptors on the cell surface.

We tested all 85 CD4 T-cell transductants for a response to islet peptides using a bioassay in which a T-cell transductant secretes IL-2 upon antigen stimulation. Peptides for these functional assays came from overlapping preproinsulin peptides (Fig. 3*B*), insulin B:9–23, and three well-defined mimotopes from B:9–23

(19,35), GAD 555–567 (36), and chromogranin A (37), which were determined from reactivity in peripheral blood of patients with new-onset type 1 diabetes (Fig. 3*C*). T-cell transductants were cultured with peptides in the presence of murine B-cell lines (33) expressing human HLA-DR3, DR4, DQ2, DQ8, and DQ2-trans (composed of the DQ8 α-chain, and DQ2 β-chain) or DQ8-trans (DQ2 α-chain and DQ8 β-chain) as antigen-presenting cells based upon the HLA typing of each patient. Screening of the 85 T-cell transductants identified eight T cells exhibiting moderate to strong responses (Fig. 3*A*). Remarkably, three of the responders were specific for preproinsulin peptides; the other five T cells responded to a DQ or DR molecule likely expressing an endogenous peptide derived from antigen-presenting cells, because responses were not dependent upon in vitro added peptide. These five T-cell transductants were tested using Epstein Barr virus–transformed B-cell lines having the DR3-DQ2/DR4-DQ8 genotype to present islet peptides, which removed the nonspecific stimulation and revealed that none of the transductants respond to the tested peptides (Supplementary Fig. 4). T-cell transductant GSE.8E3 responded to C-peptide amino acids 19–35 (C:19–35) in the presence of DQ2-trans and DQ8-trans antigen-presenting cells (Fig. 3*A* and Supplementary Fig. 5). Experiments to determine HLA restriction revealed that GSE.8E3 responds to the peptide presented by DQ8-trans (Fig. 4*A*).

The other two TCRs identified by functional screening, named GSE.6H9 and GSE.20D11 from donors 6323 and 6342, respectively, responded to the insulin B:9–23 peptide (Fig. 3*A*). Of note, these two TCRs (one or both of α-chain/β-chain) were repeatedly detected in the islets of individual donors (Supplementary Table 6). Experiments analyzing HLA restriction showed that GSE.6H9 recognized insulin B:9–23 presented by both DQ8 and DQ8-trans (Fig. 4*B*) with response to the peptide presented by DQ8 nearly three orders stronger than presentation by DQ8-trans (Supplementary Fig. 6*B*). This TCR is cross-reactive with the same peptide presented by HLA molecules sharing the same DQ β-chain but having different DQ α-chains. The second B:9–23–specific T-cell transductant, GSE.20D11, was identified from a different organ donor (donor 6342) and responded to B:9–23 presented by DQ8 (Fig. 4*C*). The levels of response to B:9–23 were dose dependent but were not as robust as that by a T-cell transductant reacting with a neoantigen or deamidated α-gliadin (38), and were several orders of magnitude stronger than the GSE.8E3 response to C:19–35 (Supplementary Fig. 6*A*, *C*, and *D*). To determine the core amino acid sequence required for the two TCRs to recognize B:9–23, we examined the responsiveness to series of truncated B:9–23 peptides (Supplementary Fig. 7). The minimum requirement for both GSE.6H9 and GSE.20D11 was insulin B-chain amino acids 12–22. Last, for all three proinsulin-reactive TCRs, dose-dependent responses by T-cell transductants expressing native human CD4 in place of mutated human CD4 were confirmed (Supplementary

Table 2—T-cell isolation and TCR sequencing from pancreatic islets of organ donors with type 1 diabetes

	nPOD donor 6323			nPOD donor 6342			nPOD donor 69	
Islets studied (*n*)	1,550 handpicked			500 IEQ			500 handpicked	
Time from death prior to receiving islets (days)	2			2			7	
Duration of tissue culture (days)	3–4			1–3			4	
Cells for flow cytometry analysis (*n*)	Day 3: 508,239 Day 4: 789,254			Day 1: 1,254,156 Day 3: 1,512,140			3,118,311	
	CD4	CD8		CD4	CD8		CD4	CD8
Cells detected, *n* (% of total cells acquired)	Day 3: 398 (0.078%) Day 4: 1,631 (0.207%)	Day 3: 1,091 (0.215%) Day 4: 4,472 (0.601%)		Day 1: 1,985 (0.158%) Day 3: 2,657 (0.176%)	Day 1: 1,389 (0.111%) Day 3: 1,341 (0.089%)		29 (0.0009%)	141 (0.0045%)
Total cells isolated for TCR sequencing (*n*)	393	177		507	240		12	58
Cells identified α-chain sequences (*n*)	236	120		328	154		10	39
Cells identified β-chain sequences (*n*)	277	144		415	181		7	42
No. with TCR α- and β-chains	189	103		295	129		7	34
TCRs studied for preproinsulin reactivity (*n*)	40	Not done		43	Not done		7	Not done

IEQ, islet equivalent.

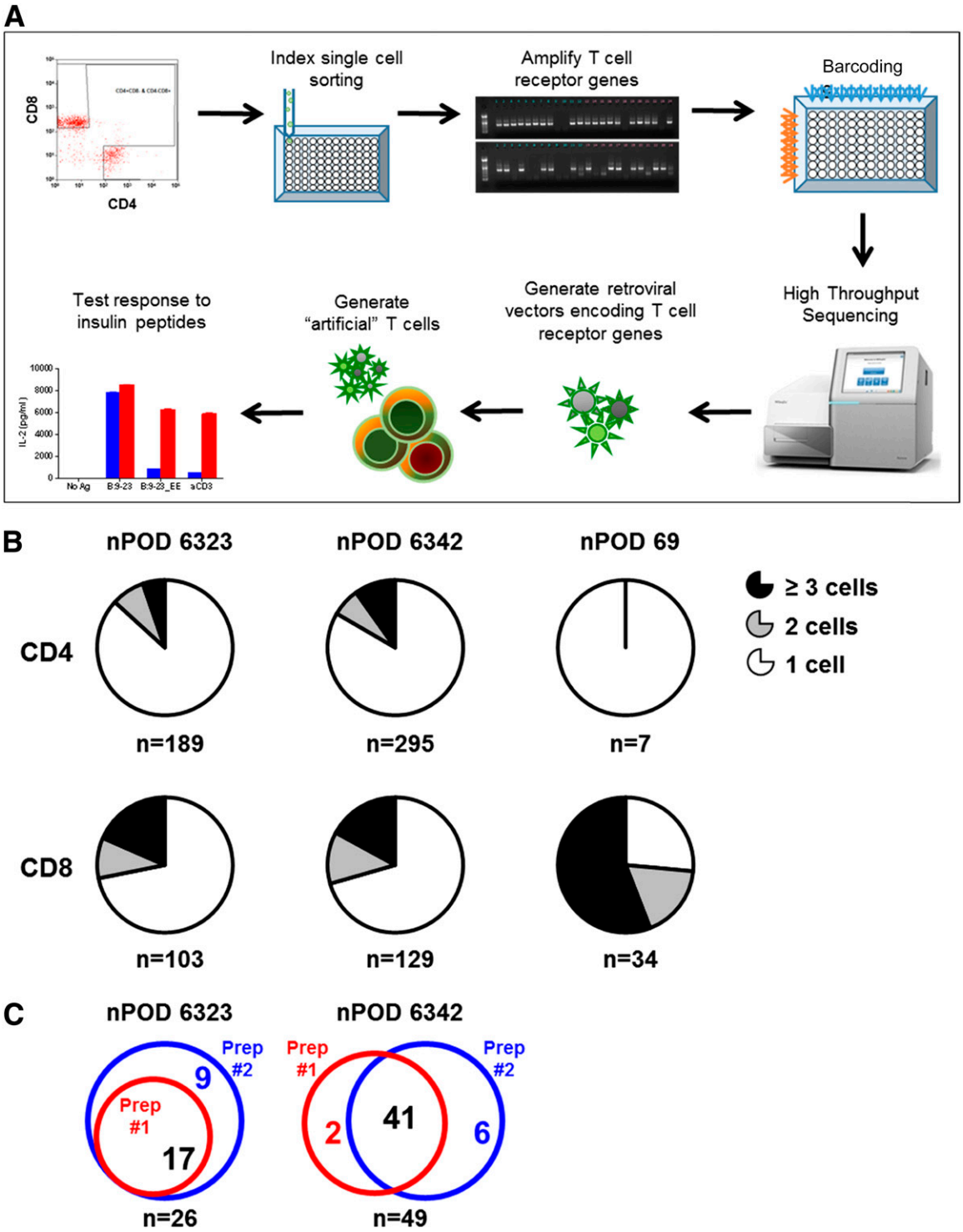
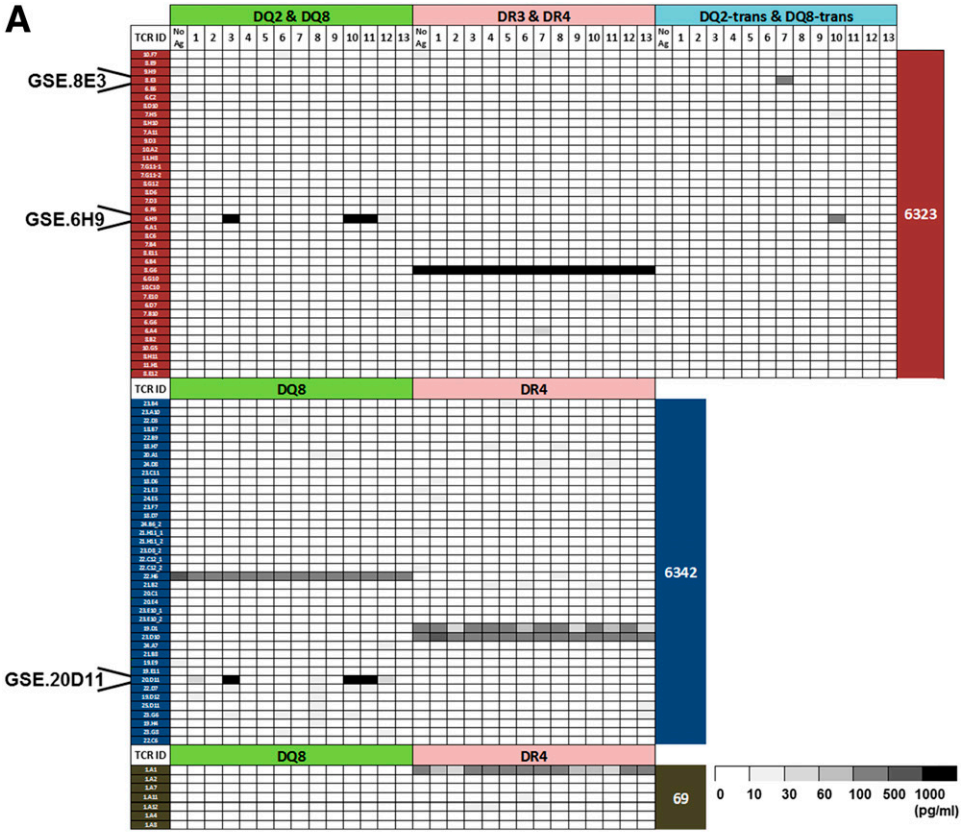
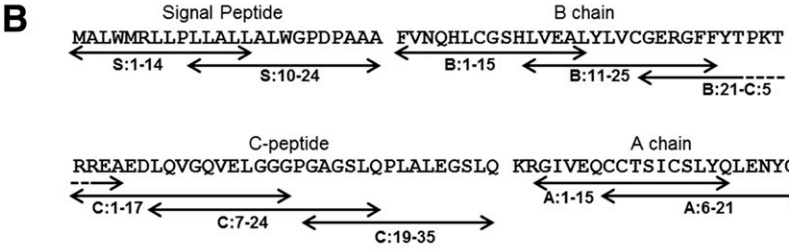


Figure 2—TCR sequencing of T cells in the pancreatic islets of organ donors with type 1 diabetes. *A*: Strategy to test the antigen specificity of T cells infiltrating pancreatic islets. CD4 and CD8 T cells were isolated from islets by single-cell sorting, followed by amplification and sequencing of individual TCRs. "Artificial" T cells, termed T-cell transductants, were generated using retroviral vectors encoding the detected TCR sequences of interest such that immortalized cells were made expressing only a single TCR. The TCR transductants were then tested for a functional response to candidate antigens, including overlapping peptides derived from preproinsulin. *B*: Diversity of TCR sequences of T cells infiltrating pancreatic islets in the organ donors with type 1 diabetes. Several to hundreds of TCR α and β paired sequences were determined from each individual donor. White represents sequences detected only once, gray twice, and black three or more times. CD4 TCR sequences exhibit more diversity, whereas CD8 repertories have more clonality than CD4 in all three patients. *C*: TCR sequences detected two or more times in separated islet preparations from a single patient. CD4 T cells for single-cell analysis were isolated from two separate islet preparations for donors 6323 and 6342. Of TCR sequences detected repeatedly, the numbers of those detected in each islet preparation are shown. The majority of repeatedly detected TCRs were found from multiple islet preparations in the same patient.





C

Peptide Number	Islet Protein	Name	Amino Acid Sequence
1	Preproinsulin	S:1-14	MALWMRLLPLLALL
	Preproinsulin	S:10-24	LLALLALWGPDPAAA
2	Preproinsulin	B:1-15	FVNQHLCGSHLVEAL
3	Preproinsulin	B:11-25	LVEALYLVCGERGFF
4	Preproinsulin	B:21-C:5	ERGFFYTPKTRREAE
5	Preproinsulin	C:1-17	RREAEDLQVGQVELGGG
6	Preproinsulin	C:7-24	LQVGQVELGGGPGAGSLQ
7	Preproinsulin	C:19-35	GAGSLQPLALEGSLQKR
8	Preproinsulin	A:1-15	GIVEQCCTSICSLYQ
9	Preproinsulin	A:6-21	CCTSICSLYQLENYCN
10	Preproinsulin	B:9-23	SHLVEALYLVCGERG
11	Preproinsulin	B:9-23 (B22E)	SHLVEALYLVCGEEG
12	Chromogranin A	pS3 (p1E)	SELGLWVRME
13	Glutamic Acid Decarboxylase	GAD555-567 (557I)	NFIRMVISNPAAT

Figure 3—Screening of islet-infiltrating TCR transductants for response to preproinsulin and islet peptides. (*A*) Heatmap depicts antigen-stimulated T-cell transductants derived from receptor sequencing of CD4 T cells. Responses are shown for each individual T-cell transductant, measured by IL-2 secretion, to overlapping preproinsulin peptides (*B*) and all tested islet peptides (*C*) in the presence of a mixture of antigen-presenting cells expressing DQ2 or DQ8, DR3 or DR4, or DQ2-trans or DQ8-trans. Each row denotes an individual TCR transductant with 38 tested from donor 6323, 40 from donor 6342, and 7 from donor 69. The peptide number in the columns of the heatmap corresponds to those in panel *C*. Three T cells, named GSE.8E3, GSE.6H9, and GSE.20D11, responding to proinsulin peptides were identified.

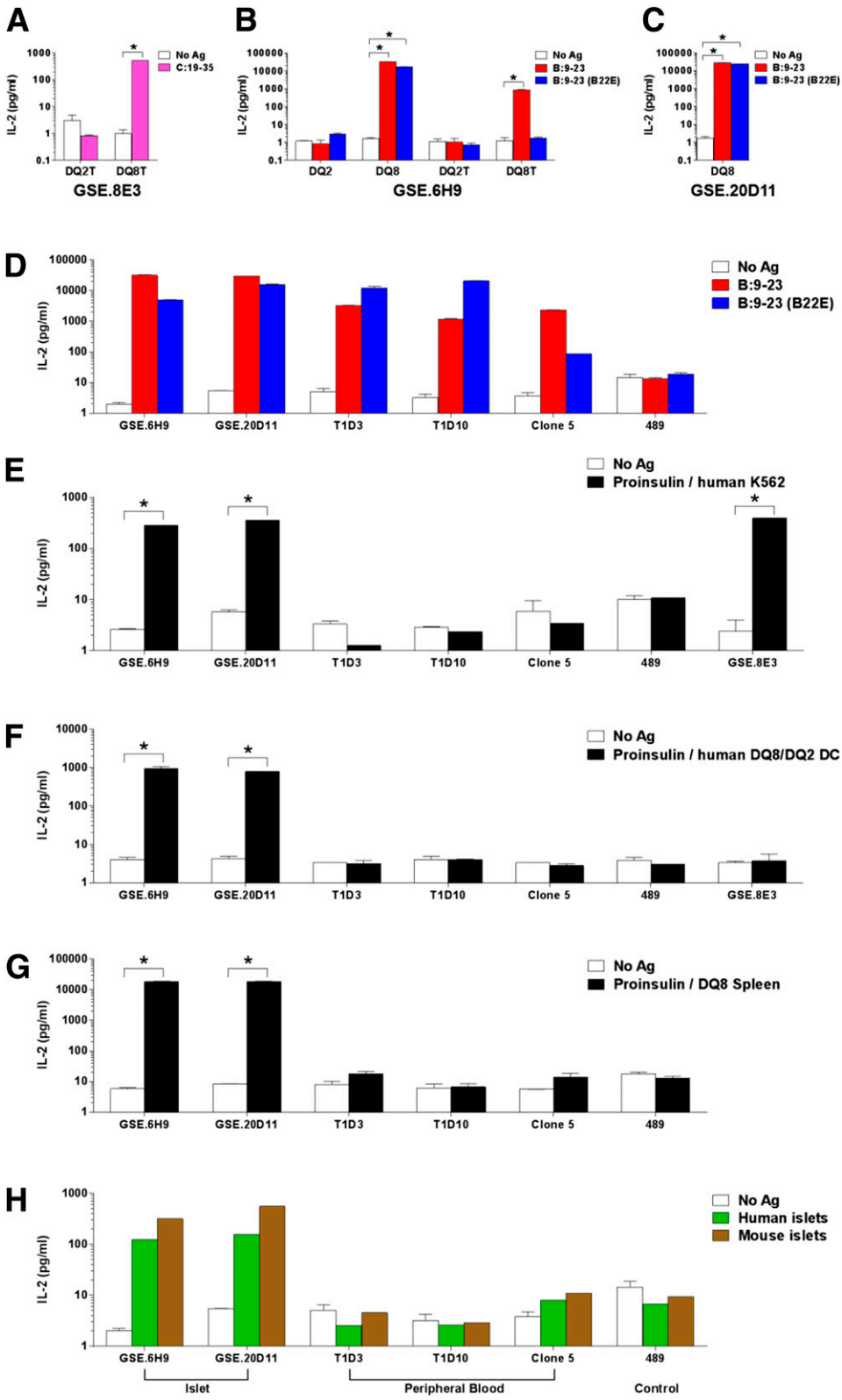


Figure 4—Response to antigens and islets by insulin B:9–23–reactive T cells identified within pancreatic islets and peripheral blood. T-cell transductants GSE.8E3 (*A*), GSE.6H9 (*B*), and GSE.20D11 (*C*) were cultured with cognate peptides C:19–35 for GSE.8E3 (*A*) or insulin B:9–23 and its mimotope B:9–23 (B22E) for GSE.6H9 and GSE.20D11 (*B* and *C*) in the presence of murine B cell lines expressing DQ2, DQ8, DQ2-trans, or DQ8-trans to determine HLA restriction. *D*–*G*: T-cell transductants expressing proinsulin-reactive TCRs derived from the islets (GSE.8E3, GSE.6H9, GSE.20D11), those previously cloned from peripheral blood of other patients with type 1 diabetes (T1D3, T1D10, and Clone 5), or an α-gliadin–responsive TCR (489) as a control were tested for reactivity to proinsulin, islets, and insulin B:9–23 peptides. The T-cell transductants were cultured with insulin B:9–23 peptides (*D*), whole proinsulin in the presence of human monocyte-derived

Fig. 8), demonstrating that the responses were not due to two mutations within the CD4 molecule. In sum, screening 85 TCRs derived from islet-infiltrating CD4 T cells for the response to islet antigens revealed three proinsulin specificities presented by DQ8/DQ8-trans, two of which that were derived from separate individuals were insulin B:9–23 specific. GSE.6H9 and GSE.20D11 are the first insulin B:9–23–reactive CD4 T cells identified from inflamed pancreatic islets of patients with type 1 diabetes.

Reactivity to Whole Proinsulin and Islets

Finally, we tested the three proinsulin-reactive TCRs for response to whole proinsulin protein and pancreatic islets presented by DQ8-bearing antigen-presenting cells. All the proinsulin-reactive T-cell transductants responded to whole proinsulin in the presence of the K562 human myelogenous cells expressing the cognate HLA DQ molecule (i.e., DQ8 or DQ8-trans) (Fig. 4*E*). When using human monocyte–derived dendritic cells from a DQ2/DQ8 individual, only the B:9–23–reactive GSE.6H9 and GSE.20D11, but not GSE.8E3, T-cell transductants responded to whole proinsulin, presumably because of insufficient antigen presentation by DQ8-trans (Fig. 4*F*). GSE.6H9 and GSE.20D11 also responded to whole proinsulin when cultured with murine DQ8 transgenic splenocytes in a dose-dependent manner (Fig. 4*G* and Supplementary Fig. 6*B* and *C*), indicating that insulin B:9–23 is processed and presented from proinsulin by both human and murine antigen-presenting cells. Importantly, GSE.6H9 and GSE.20D11 responded to single cells dispersed from human and murine pancreatic islets in the presence of DQ8 transgenic splenocytes (Fig. 4*H*).

For comparison, we examined B:9–23–reactive T cells that have been cloned previously from the peripheral blood of patients with type 1 diabetes (19,20,39), separate from the organ donors studied. T-cell transductants expressing TCRs from these T-cell clones were generated using the same conditions as for the islet-derived TCRs, such that all transductants were identical except for the TCR sequence. The two islet-derived B:9–23 TCRs (GSE.6H9 and GSE.20D11) secreted 5–10 times more IL-2 upon peptide stimulation compared with the peripherally derived B:9–23 TCRs (T1D3, T1D10, and clone 5) (Fig. 4*D*). Interestingly, the peripheral blood–derived TCR transductants did not respond to proinsulin (Fig. 4*E–G*) or islets (Fig. 4*H*). Despite the fact that all five T cells are insulin B:9–23 reactive, only islet-derived GSE.6H9 and GSE.20D11 responded to proinsulin and islets, indicating important differences between B:9–23–specific T cells based upon tissue location.

DISCUSSION

Using a direct isolation approach guided by single-cell TCR sequencing, we determined in vivo TCR repertories and antigen specificity from T cells in inflamed pancreatic islets from limited tissue samples of young organ donors with type 1 diabetes having residual insulin-positive islets. Among 85 CD4 TCRs analyzed, two detected from separate patients were reactive to insulin B:9–23. The third T cell responded to C:19–35 presented by DQ8-trans. Notably, responsiveness to whole proinsulin and islet cells was confirmed for the insulin B:9–23–reactive T cells derived from pancreatic islets but not those from peripheral blood, indicating the importance of studying T cells from the islet microenvironment. Insulin B:9–23 is an essential self-antigen–determining disease development in the NOD mouse model (12–14), which shares homology with human type 1 diabetes in terms of structurally similar MHC class II molecules (40,41) and identical B:9–23 amino acid sequences (10). Both of the highest-risk alleles, human HLA-DQ8 and NOD I-A^{g7}, have an amino acid polymorphism at position 57 of the β-chain, rather than a conserved aspartic acid in diabetes nonrisk alleles, resulting in similar peptide repertoires, preferably presented by these MHC molecules (42). Further, our results are reminiscent of work from the NOD mouse in which type A insulin B:9–23 T cells respond to both peptide and insulin protein, which are highly deleted in the thymus, whereas type B T cells respond only to peptide and are activated in the periphery (43). Our current findings provide the first direct evidence that insulin B:9–23 is targeted by T cells present within the pancreatic islets of patients with type 1 diabetes, and, importantly, these T cells respond to whole proinsulin and islets presented by antigen-presenting cells bearing HLA-DQ8 and DQ8-trans.

There is a report analyzing CD4 T cells cloned from pancreatic islets of a single patient with type 1 diabetes that identified two peptides within C-peptide as their targets (24). We confirmed the reactivity to C:19–35 from a separate patient with type 1 diabetes in which only one of our three donors had the appropriate alleles to form DQ8-trans. Thus, amino acids 19–35 of C-peptide may be commonly recognized by T cells within the islets of patients with type 1 diabetes having high-risk HLA-DQ8 and DQ2 genes allowing the formation of the DQ8-trans molecule. Importantly, our current analysis detected response to whole proinsulin by the C:19–35–reactive T cell, further supporting the involvement of T cells

dendritic cells derived from a DQ8/DQ2 heterozygous individual (*E*), whole proinsulin in the presence of the K562 human myelogenous cells expressing DQ8 (for all other than GSE.8E3 TCRs) or DQ8-trans (for GSE.8E3) (*F*), whole proinsulin in the presence of DQ8-transgenic spleen cells (*G*), islets isolated from T cell–deficient NOD mice, and human organ donors without diabetes in the presence of DQ8-transgenic spleen cells (*H*). Levels of IL-2 secreted into the tissue culture supernatant were measured by a highly sensitive ELISA. Distinct differences in response to whole proinsulin and islets are noted between insulin B:9–23–reactive T cells based upon the originating tissue location. All data shown are representative of at least three independent experiments. $^*P < 0.01$ by two-tailed unpaired *t* test. Ag, antigen.

targeting this peptide in anti-islet autoimmunity in patients with type 1 diabetes.

The method we used to directly isolate and sequence single TCRs from the islet infiltrate allows us to recapitulate the in vivo T-cell repertoire in the pancreas due to a short in vitro culture time. With this unique approach, the frequency of proinsulin-reactive T cells identified in the current study was 3 of 85 TCRs (3.5%). In the NOD mice, insulin B:9–23–reactive T cells are typically <5% in inflamed pancreatic islets as measured with fluorescent tetramers (44), although tetramers may not detect low-avidity T cells that are reactive with B:9–23 and likely underestimates the true frequency of these cells. Alternative analysis using the single-cell TCR sequencing (a similar approach was used in the current study) shows slightly more abundant B:9–23–reactive T cells in prediabetic NOD islets (10–15%; unpublished data), but B:9–23–reactive T cells are still a minor population. Despite this frequency, the B:9–23 epitope is essential for diabetes development in the NOD. The indispensability of proinsulin peptides defined here (insulin B:9–23 and C:19–35) to initiate or drive anti-islet autoimmunity in humans needs to be explored in future studies.

The current study now raises several questions. First, although the three donors analyzed had a relatively short duration of diabetes, it will be important to analyze individuals with diabetes (i.e., those developing multiple islet autoantibodies but no overt hyperglycemia) to understand the pathogenic mechanism by which T cells initiate islet autoimmunity. If insulin B:9–23–reactive T cells are as important in the development of diabetes in humans as in the NOD mice, B:9–23–specific T cells are predicted to be more prominent in the pancreatic islets of patients with prediabetes. In our study, all three donors received subcutaneous insulin injections for treatment, and therefore we cannot completely exclude the possibility that the B:9–23–specific T cells were activated by exogenous insulin. However, these T cells were isolated from inflamed islets and not peripheral blood, and, despite screening responsiveness to overlapping insulin peptides, no responses to other insulin peptides were identified, indicating the importance of insulin B:9–23. Additionally, the islet-derived B:9–23–specific T cells reacted with whole proinsulin and islets, further highlighting the significance of these cells.

Second, although ~60% of patients with type 1 diabetes have the high-risk HLA-DR4-DQ8 genes (45), it remains to be addressed whether insulin B:9–23 presented by other HLA molecules is a target for islet-derived T cells. None of our studied TCR transductants responded to B:9–23 presented by DQ2, DQ2-trans, or DR4 molecules.

Last, antigens targeted by the remaining T cells identified in the current study are still unknown. We were only able to determine antigen specificity for 3 of 85 CD4 T cells studied, which may indicate a low frequency of proinsulin-reactive T cells within the islets of patients with short-term type 1 diabetes. Alternatively, our approach of using T-cell transductants in which chimeric TCRs were reconstituted in murine T-cell hybridomas could underestimate the antigen specificity of these T cells. It is also a distinct possibility that the optimal epitopes for the remaining T cells have not yet been determined. Other peptides, including those within GAD, islet antigen-2, and zinc transporter-8, may be targets for the remaining T cells. In particular, an unknown post-translational modification may be required to form the optimal epitopes that stimulate the remaining T-cell transductants robustly enough. Evidence for post-translational modification comes from recent reports that autoreactive T cells can target hybrid peptides formed by linking a fragment of proinsulin with peptides derived from other proteins within β-cells, potentially through a process of transpeptidation (46), whereas another report (47) identified a deamidated proinsulin epitope as the target of proinflammatory T cells of patients with type 1 diabetes. High-throughput screening strategies to determine the response to human islets and islet-derived peptides will be required to characterize the targets of our remaining T cells, including CD8 T-cell targets presented by HLA class I molecules.

Several randomized clinical trials (48–50) have focused on using insulin preparations, including an altered peptide ligand of B:9–23 as antigen-specific therapy to prevent type 1 diabetes onset, with relatively disappointing results. The Diabetes Prevention Trial-Type 1 conducted two concomitant studies using parenteral insulin and oral insulin in relatives who were at risk for type 1 diabetes. Diabetes onset was not delayed in either treated cohort compared with control arms (4). However, a post hoc analysis of participants treated with oral insulin revealed a 5-year delay in diabetes onset when high titers of insulin autoantibodies were present at study enrollment, prompting a repeat trial (5). Using higher doses of oral insulin earlier in the course of disease induced protective immune responses to insulin, as evidenced by recent results from a pilot trial (51) in children genetically at risk for type 1 diabetes without autoantibodies. Dose, route, timing in the disease process, and a threshold immune response to insulin appear to be critical for future type 1 diabetes prevention efforts. Our findings identify disease-relevant proinsulin-responsive T cells, providing the framework to monitor and target these cells. In particular, given evidence that insulin B:9–23 is a primary autoantigen determining disease progress in the NOD mouse (12–14), our current finding of B:9–23 as a target antigen for T cells within the pancreatic islets of patients with type 1 diabetes provides a framework to develop more targeted and robust therapies to modify the autoimmune response to insulin.

In conclusion, we report direct evidence that proinsulin epitopes insulin B:9–23 and C:19–35 are targets for T cells within inflamed pancreatic islets of patients

with type 1 diabetes. This is the first report that insulin B:9–23 is a target for islet-derived CD4 T cells. These T cells show distinct responses to whole proinsulin and islets, indicating the importance of studying T cells from the target organ in human disease. We believe these findings provide a long-standing hallmark to understand human autoimmune diabetes pathogenesis that will lead to improved therapies to prevent type 1 diabetes.

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Author Contributions. A.W.M. and M.N. designed the research studies, conducted the experiments, acquired and analyzed the data, and wrote and edited the manuscript. L.G.L. conducted the experiments and acquired and analyzed the data. K.A.M. conducted the experiments and acquired the data. L.Y. analyzed the data and provided essential reagents. M.C.-T. conducted the experiments, acquired and analyzed the data, and wrote and edited the manuscript. W.W.K. provided essential reagents. K.L.J. analyzed the data. P.A.G., Q.T., M.A.A., and C.E.M. designed the research studies and wrote and edited the manuscript. J.W.K. designed the research studies. B.O.R. provided essential reagents and wrote and edited the manuscript. M.N. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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